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Selective detection of rapid motions in spectrin by NMR

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Human erythrocyte spectrin molecules exhibit relatively sharp (30–50 Hz) proton NMR signals in the aliphatic region. A standard solvent presaturation pulse sequence that also partially suppresses the broad envelope from protons with rigid structures in spectrin and selectively enhances the sharp resonances has been used to characterize the behavior of these resonances. The overall resonance pattern strongly resembles that of the denatured spectrin. The observed spectra are also quite similar to the line-broadened spectrum from a mixture of amino acids that corresponds to the composition of the spectrin molecule. These data indicate the existence of regions exhibiting rapid internal motions within the intact spectrin molecule, and suggest that the amino acid composition of the residues giving rise to the sharp resonances is quite similar to that of the full spectrin molecule.

Erythrocyte membrane *Membrane skeleton* *Spectrin-actin complex* *Spectrin* *Internal motion* *NMR*

1. INTRODUCTION

Spectrin, a heterodimeric protein of 460 kDa, is a major component of the human erythrocyte membrane skeleton [1]. The membrane skeleton is believed to maintain the biconcave shape of normal cells [2], and to control the lateral mobility of intrinsic membrane proteins [3]. Some of the physical and chemical properties of the spectrin molecule have been extensively studied [4]. In comparison, however, little is known about the molecular dynamics of spectrin, either in the intact skeletal network, or in solution. To understand the function of proteins requires an investigation of the dynamics of protein structural fluctuations [5,6], and their relation to specific conformational changes.

We have previously used saturation-transfer EPR techniques to study the flexibility of spectrin and have shown that the motions of spectrin in the spectrin-actin network include multiple classes or rates of motion. The spin-labeled spectrin-actin complex exhibits 3 principal motional components, with rotational correlation times of less than 10^{-9} s, 10^{-7}–10^{-6} s, and about 10^{-3} s [7]. NMR techniques have been used very successfully in studying different aspects of the dynamics of proteins of moderate molecular mass, but have not often been used for proteins with very high molecular mass (above 100 kDa) due to broad, overlapping signals with low resolution [8]. However, recent development in the use of various pulse sequence techniques in FT-NMR eliminates some of the broad envelope to provide observable sharp resonances (about 30–50 Hz) in proton NMR spectra of such very large proteins as myosin, the pyruvate dehydrogenase complex, and immunoglobulins [9–12]. These pulse sequence techniques generate cross-relaxation and spin diffusion in proteins with consequent non-specific nuclear Overhauser effect [13–16]. An early paper revealed some sharp resonances in the proton NMR spectrum of an ammonium sulfate-precipitated sample of spectrin; however very little discussion of the NMR data was given [17].

We have now used a simple, standard solvent presaturation method to investigate further the molecular motions of the native spectrin molecule,

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both in the spectrin-actin network, and in the purified, dimeric form. The proton NMR spectra exhibit several sharp resonances, and differ from those published earlier [17]. Our NMR spectra of native and denatured spectrin and spectrin amino acid mixture are similar in chemical shifts, but differ substantially in absolute intensity, suggesting that the mobile segments of the spectrin molecule have a random coil structure.

2. MATERIALS AND METHODS

The spectrin-actin complex was solubilized from human erythrocyte white membrane ghosts in 0.3 mM phosphate buffer at pH 7.6. Spectrin was further purified from actin with a Sepharose 4B-CL column and Tris buffer [18]. Fractions from the spectrin dimer peak were pooled. SDS gel electrophoresis showed only bands 1 and 2, with no trace of any other bands (fig.1A), and non-SDS gel electrophoresis [19] indicated about 90% dimer and 10% tetramer (fig.1C). The pooled spectrin sample and the spectrin-actin complex sample were concentrated with an ultrafiltration cell and an Amicon XM100 membrane, dialyzed with 5 mM phosphate buffer at pH 7.4. The samples were then exchanged with deuterated phosphate buffer containing 150 mM NaCl at pH 7.4 using a 30 kDa cut-off micro-concentrator from Amicon, to give less than 5% residual water content in samples, and used as NMR samples. The concentrations of these samples were 4 mg/ml, as measured by the Lowry assay. SDS gels on samples after the NMR experiments showed bands 1 and 2, as expected, and less than 1–2% of various lower molecular mass (but larger than 100 kDa) bands, along with a very high molecular mass band (fig.1B). Similar gel data were obtained on samples in buffers containing 0.1 mM NaN_3 and 0.01 mM phenylmethanesulphonyl fluoride. Non-SDS gel data on NMR samples showed about 75% dimers and 25% tetramers (fig. 1D). The increased amount of tetramer was mainly due to the relatively high concentration of spectrin in the NMR sample. The amino acid mixture sample was prepared in deuterated buffer, following the amino acid composition of spectrin [20]. Concentrated NaOD was added directly to spectrin samples in deuterated buffer to give the NaOD-treated samples. Circular dichroism (CD) measurements were made on the intact and NaOD-treated spectrin samples with a Jasco J-40A spectropolarimeter. Ellipticities in the range 200–270 nm were measured. The α-helical content of each sample was estimated by linear regression analysis of the spectrum, using the spectra of various peptide secondary structures as a basis as derived by Chen et al. [21]. The proton NMR spectra were obtained at 37°C on a 200 MHz Nicolet NMR spectrometer with a solvent presaturation pulse sequence, which consisted of a 2 s solvent irradiation period, followed by a 70° (6 μs) pulse, followed by acquisition in 4K memory; total recycle time, including acquisition time, was 2.7 s. The spectral width was 15 ppm. NMR experiments were done on samples within 5 days after membrane preparation.

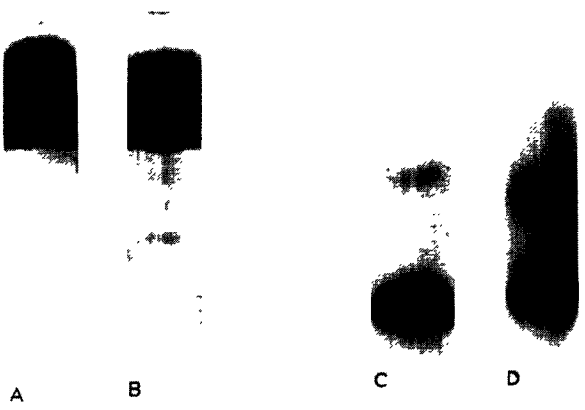

Fig.1. (A) SDS gel (5.6% polyacrylamide) electrophoresis of spectrin dimer fraction from Sepharose 4B-CL column (about 0.5 mg/ml); (B) SDS gel of concentrated spectrin NMR spectrin sample (4 mg/ml) (see text) with less than 1% smaller molecular mass bands. (C) Non-SDS gel (2.5% acrylamide and 0.3% agarose composite gels) of spectrin sample as used in (A), with 90% dimers and 10% tetramers. (D) Non-SDS gel of spectrin sample as used in (B) with about 75% dimers and 25% tetramers.

3. RESULTS

The presaturation proton NMR spectra of spectrin in the spectrin-actin network (fig.2A) and in purified form (fig.2B) exhibit several relatively sharp resonances in the aliphatic region, about 1.5–4.5 ppm upfield from the HDO resonance; the linewidth (half-height full width) of the major

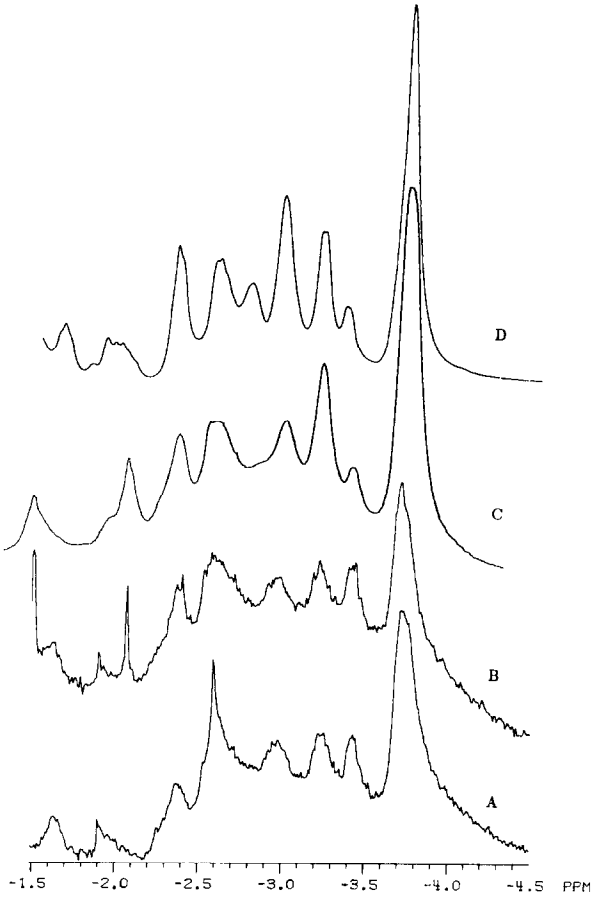

Fig.2. 200 MHz proton NMR spectra obtained by using a presaturation pulse sequence with the HDO resonance being irradiated for 2 s first, followed by a 2 ms delay, and then a 70° (6 μs) pulse, followed by a 0.7 s acquisition time, and a 10 ms post-delay. Spectra of spectrin-actin complex (4 mg/ml) (A) and of purified spectrin (B), both in deuterated 5 mM phosphate buffer with 150 mM NaCl at pH 7.4 with less than 5% residual water content. (C) The same spectrin sample as in (B) except NaOD was added prior to the NMR experiment. A 1 Hz line broadening was used for spectra A–C. (D) An amino acid mixture with a composition equivalent to the spectrin amino acid composition. A 10 Hz line broadening was used for spectrum D to broaden artificially the resonance linewidths to match with those in (A–C).

peak at −3.7 to −3.8 ppm was estimated to be about 25–35 Hz. These two spectra were remarkably similar, both in chemical shifts, and in the relative intensities of the resonances. Non-SDS gels of purified spectrin samples showed about

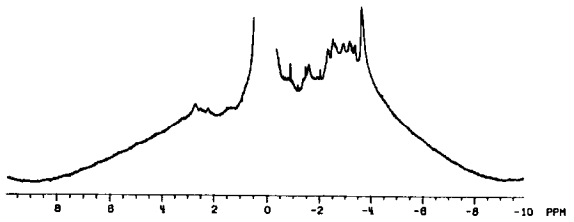

Fig.3. 200 MHz proton NMR spectrum of spectrin, in phosphate buffer with 150 mM NaCl, obtained by using a one-pulse method with 15 s delay time and 20 ppm sweep width, and no water presaturation.

75% dimers and 25% tetramers, with the CD measurements indicating about 85 ± 9% α-helical content. Spectrum D in fig.2 of NaOD-treated spectrin is also generally similar in the chemical shifts and relative intensities to the spectra of native spectrin (spectra A and B). However, the overall integrated intensity of the resolved signals in spectra A and B is only about 10% of that in spectrum C. CD data showed only about 24 ± 3% α-helical content in the NaOD-treated sample, indicating that most of the α-helical structure in native spectrin has become random coil upon addition of NaOD. Spectrum D in fig.2 of the amino acid mixture also exhibits strong similarities to spectra A–C. A 10 Hz line broadening was applied to this spectrum to match artificially its linewidths with those in the spectra of spectrin molecules.

4. DISCUSSION

Calvert et al. [17] have published an NMR spectrum of ammonium sulfate-precipitated spectrin sample that exhibits several sharp resonances, and suggested the existence of segmental motions in spectrin. They further suggested that the amino acids that gave rise to their NMR signal in spectrin did not reflect the amino acid composition of the whole protein. The suggestion of segmental motions in spectrin is interesting. However, the lack of detailed information about the results leads to several questions. The spectrin molecule is a relatively large molecule, but its published NMR spectrum shows no broad envelope that would normally be expected from such a large molecule. Generally, sharp resonances are associated with protons in small molecules, in denatured molecules, or in impurities. What is the integrity of

the 'native' spectrin sample used to obtain the published NMR spectrum?

Here, we have taken great care in spectrin preparation. To ensure that the sharp resonances observed for spectrin are from the native protein, we have designed our sample preparation procedures to minimize the presence of low molecular mass components (less than 30 kDa) and of denatured molecules. We have avoided harsh protein treatments, such as ammonium sulfate precipitation, in order to preserve the integrity of spectrin. Furthermore, we have used both non-SDS gel electrophoresis and CD measurements to ascertain the 'nativeness' of the spectrin molecules used in the NMR experiments. In addition, our NMR spectra of the isolated spectrin-actin complex exhibit a remarkable resemblance to that of purified spectrin. Thus, we believe that the relatively sharp resonances arise from spectrin molecules in the native conformation, and not from denatured spectrin or impurities in the samples. Our single-pulse spectrum of the native spectrin with no irradiation of the HDO resonance exhibits a broad envelope underlying the sharp resonances (fig.3). Using a standard water presaturation pulse sequence, we selectively enhance the sharp resonances in native spectrin samples. Irradiation at the HDO resonance also results in saturation of the backbone CH proton resonances. In general, the relaxation of the backbone α protons and of the side-chain protons that are held rigidly in the molecular framework of large proteins is dominated by cross-relaxation [8,13,16]. When spin diffusion is prevalent, the nuclear Overhauser effect becomes completely nonspecific, and continuous irradiation of any of the protons in the rigid part results in saturation of all other resonances corresponding to rigid nuclei. However, spin diffusion extends much more slowly, and thus less effectively, to the flexible parts of the protein with rapid internal motions. Thus we believe that the relatively sharp resonances shown in fig.2A and B arise from regions within the intact spectrin molecules that exhibit segmental motions.

The expected linewidths due to methyl group rotation at rates either faster or slower than the protein rotational correlation time are about 60 and 170 Hz for a molecular mass of 100 kDa, and 170 and 670 Hz for 450 kDa, respectively, based on the Woessner model of internal motions in proteins [9]. Our observed signals are about 25–35 Hz, which is substantially less than the expected lower limit of the linewidths. The overlap of several resonances with linewidths even narrower than 25–35 Hz may also give apparent linewidths of 25–35 Hz. Our NMR data clearly indicate that the portions of spectrin giving rise to the sharp resonances (with apparent linewidths of 25–35 Hz) are undergoing rapid internal motions.

The sharp resonances in the spectra in fig.2 appear to differ from those in [17]. Our spectra of spectrin in phosphate buffer, of NaOD-denatured spectrin, and of the amino acid mixture for spectrin all show very similar resonance patterns, suggesting that the segments of the molecules exhibiting rapid internal motion have an amino acid composition very similar to that of the full protein, in contrast to the previously published work that suggests that some types of amino acids may be selectively depleted from the mobile segments [17]. The spectra of fig.2 exhibit only minor differences; for example, a resonance at about -2.8 ppm (the position of the Arg βCH_2 groups) that is observed in fig.2D is missing in fig.2B. The observation that the pattern of resolved resonances for native spectrin closely resembles that expected from its overall composition appears rather remarkable, in that one might expect to see a predominance of resolved aliphatic resonances from the side chains of such polar residues as Lys, Arg, Asp, Glu or Gln, but a reduced contribution from the side chains of such nonpolar residues as Leu, Val, Ile or Ala, due to the greater probability of the polar residue side chains projecting freely out of the protein into the solvent, and the nonpolar side chains being 'trapped' within the protein structure. However, the methyl resonance at about -3.8 ppm, which must be produced by the side chains of the nonpolar residues, is not reduced in relative intensity, indicating that polar and nonpolar residues both contribute to the resolved resonances in proportion to their overall amino acid composition.

The NMR data show that the internal motions associated with these resonances are comparable to those in random coils (spectrin in NaOD). It is interesting to note that the native myosin molecule also exhibits internal motions similar to those in random coils of myosin [9]. CD and electron microscopy studies suggest that the spectrin molecule has a high helical content, and a rather

flexible structure [1,18]. Our NMR studies provide us with a means of monitoring these flexible segments in spectrin.

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REFERENCES

[1] Ralston, G.B. (1978) J. Supramol. Struct. 8, 361-370.
[2] Hainfeld, J.F. and Steck, T.L. (1977) J. Supramol. Struct. 6, 301-311.
[3] Nicolson, G.L. and Painter, R.G. (1973) J. Cell Biol. 59, 395-406.
[4] Marchesi, V.T. (1983) Blood 61, 1-11.
[5] Karplus, M. and McCammon, J.A. (1983) Ann. Rev. Biochem. 53, 263-300.
[6] Janin, J. and Wodak, S.J. (1983) Prog. Biophys. Mol. Biol. 42, 21-78.
[7] Fung, L.W.-M. and Johnson, M.E. (1983) J. Magn. Reson. 51, 233-244.
[8] Jardetzky, O. and Roberts, G.C.K. (1981) NMR in Molecular Biology, Academic Press, New York.
[9] Highsmith, S., Akasaka, K., Konrad, M., Goody, R., Holmes, K., Wade-Jardetzky, N. and Jardetzky, O. (1979) Biochemistry 18, 4238-4244.
[10] Prince, H.P., Trayer, H.R., Henry, G.D., Trayer, I.P., Dalgarno, D.C., Levine, B.A., Cary, P.D. and Turner, C. (1981) Eur. J. Biochem. 121, 213-219.
[11] Perham, R.N., Duckworth, H.W. and Roberts, G.C.K. (1981) Nature 292, 474-477.
[12] Endo, S. and Arata, Y. (1985) Biochemistry 24, 1561-1568.
[13] Akasaka, K. (1979) J. Magn. Reson. 36, 135-140.
[14] Stoesz, J.D., Redfield, A.G. and Malinowski, D. (1978) FEBS Lett. 91, 320-324.
[15] Gassner, M., Jardetzky, O. and Conover, W. (1978) J. Magn. Reson. 30, 141-146.
[16] Bothner-By, A.A. and Noggle, J.H. (1979) J. Am. Chem. Soc. 101, 5152-5155.
[17] Calvert, R., Ungewickell, E. and Gratzer, W. (1980) Eur. J. Biochem. 107, 363-367.
[18] Branton, D., Cohen, C.M. and Tyler, J. (1981) Cell 24, 24-32.
[19] Liu, S.C., Fairbanks, G. and Palek, J. (1977) Biochemistry 16, 4066-4074.
[20] Anderson, J.M. (1979) J. Biol. Chem. 254, 939-944.
[21] Chen, Y.-H., Yang, J.-T. and Chan, K.-H. (1974) Biochemistry 13, 3350.